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Nucleic Acid Sequence-Based Amplification with Oligochromatography for Detection of *Trypanosoma brucei* in Clinical Samples[▽]

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Molecular tools, such as real-time nucleic acid sequence-based amplification (NASBA) and PCR, have been developed to detect *Trypanosoma brucei* parasites in blood for the diagnosis of human African trypanosomiasis (HAT). Despite good sensitivity, these techniques are not implemented in HAT control programs due to the high cost of the equipment, which is unaffordable for laboratories in developing countries where HAT is endemic. In this study, a simplified technique, oligochromatography (OC), was developed for the detection of amplification products of *T. brucei* 18S rRNA by NASBA. The *T. brucei* NASBA-OC test has analytical sensitivities of 1 to 10 parasites/ml on nucleic acids extracted from parasite culture and 10 parasites/ml on spiked blood. The test showed no reaction with nontarget pathogens or with blood from healthy controls. Compared to the composite standard applied in the present study, i.e., parasitological confirmation of a HAT case by direct microscopy or by microscopy after concentration of parasites using either a microhematocrit centrifugation technique or a mini-anion-exchange centrifugation technique, NASBA-OC on blood samples had a sensitivity of 73.0% (95% confidence interval, 60 to 83%), while standard expert microscopy had a sensitivity of 57.1% (95% confidence interval, 44 to 69%). On cerebrospinal fluid samples, NASBA-OC had a sensitivity of 88.2% (95% confidence interval, 75 to 95%) and standard microscopy had a sensitivity of 86.2% (95% confidence interval, 64 to 88%). The *T. brucei* NASBA-OC test developed in this study can be employed in field laboratories, because it does not require a thermocycler; a simple heat block or a water bath maintained at two different temperatures is sufficient for amplification.

Human African trypanosomiasis (HAT), commonly known as sleeping sickness, is endemic in sub-Saharan Africa and is caused by the flagellate protozoan *Trypanosoma brucei*, which is transmitted by tsetse flies (*Glossina* spp.). The disease occurs in two forms, caused by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*, respectively. These subspecies are microscopically indistinguishable (26). Disease management involves screening for potential infection by the use of serological tests, checking for clinical signs, confirming the diagnosis, and staging the disease, i.e., establishing whether the patient is in the first, hemolymphatic stage or the second, encephalitic stage. These stages require different treatments, and correct identification of the stage is important for prognosis. The second stage is established by examination of the cerebrospinal fluid (CSF) obtained by lumbar puncture (27).

Conventional laboratory diagnosis entails techniques such as lymph node aspiration, blood film examination, and various more elaborate techniques to concentrate parasites in the blood (2) before microscopic detection. Microscopy has been reported to miss 20 to 30% of HAT cases, thereby denying or delaying necessary treatment (6). Early and accurate disease diagnosis is paramount, especially in HAT, where treatment depends on the stage of disease. Treatment is more effective in the early (hemolymphatic) stage of HAT, with a high recovery rate. Chemotherapy with suramin is used for *T. b. rhodesiense* HAT, and pentamidine is administered for *T. b. gambiense* HAT (18). When the trypanosomes cross the blood-brain barrier and invade the central nervous system, the disease progresses to the late (encephalitic) stage. In this stage, *T. b. gambiense* infection is preferably treated with eflornithine, but *T. b. rhodesiense* infection can be treated only with melarsoprol. Melarsoprol is a highly toxic drug and has been reported to cause death in 2 to 10% of HAT patients who receive it, due to posttreatment reactive encephalopathy (3, 26). Moreover, there is evidence of increasing drug resistance, with melarsoprol treatment failure rates of 30% reported among HAT patients in Northern Uganda (15, 16). All these shortfalls only serve to stress the urgent need for an easy, affordable, and sensitive diagnostic tool for early and accurate diagnosis of sleeping sickness.

Molecular tools such as PCR (4, 7, 13, 19) and real-time nucleic acid sequence-based amplification (NASBA) (17) have been developed for the detection of *Trypanosoma brucei* parasites. Real-time NASBA is a rapid (90-min) and isothermal (41°C) RNA amplification method, also known as "self-sustained sequence replication" (9), that has been developed for the detection of *Trypanosoma brucei* parasites (17). However, in spite of the excellent specificity and sensitivity of PCR and real-time NASBA, these methods are not commonly used in the diagnosis of African trypanosomiasis, because automated

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thermal cyclers for real-time amplification detection often are not affordable. Therefore, the identification of African trypanosomes in clinical samples still relies heavily on microscopy, a relatively insensitive method. Thus, in this study, we have developed a simple detection tool, oligochromatography (OC), to replace the real-time analyzer, making the detection of NASBA amplicons easier.

OC is a simple and rapid method of detecting nucleic acids (20) that has been used successfully after PCR for the detection of different parasites, such as *Toxoplasma gondii* (10), *Leishmania* spp. (8), *Schistosoma* spp. (1), and *T. brucei* (7). After nucleic acid amplification, the products are allowed to migrate on the sensitized membrane of an oligochromatographic dipstick. During migration, the amplified products hybridize with a capture probe and detection probes labeled with gold particles. Colored (pink/purple) lines develop after 5 to 10 min at sites of the immobilized reagent if corresponding amplicons are present. NASBA followed by OC (NASBA-OC) is a simple technique that can be introduced in diagnostic laboratories in developing regions where HAT is endemic to improve the detection of cases.

MATERIALS AND METHODS

Ethical considerations. Medical ethical clearance was provided by the Institutional Review Board of the Vector Control Division of the Ministry of Health of Uganda. Informed consent was obtained from study participants or their parents/guardians prior to their enrollment in the study.

Clinical samples. Blood (200 μl) and CSF (200 μl) were obtained from confirmed HAT patients by a medical officer for this study. HAT was confirmed by a trained laboratory technician using microscopic detection of trypanosomes in blood and/or CSF. Microscopy was done on a thick blood smear or after concentration of parasites by a microhematocrit centrifugation technique as described previously (28). In some additional cases where individuals were clinically suspected of HAT but samples were negative by microscopy, blood was examined using the mini-anion-exchange centrifugation technique to confirm the clinical suspicion (6). For microscopic detection of trypanosomes in CSF, a pellet obtained after centrifugation of 4 ml of the spinal fluid was used.

A total of 122 clinical samples (65 blood samples, of which 25 came from Uganda and 40 from the Democratic Republic of the Congo, and 57 CSF samples, 22 from Uganda and 35 from the Democratic Republic of the Congo) were included in this study. Ugandan patients came from six districts (Iganga, Bugiri, Namutamba, Soroti, Kaberamaido, and Dokolo) that are in the traditional focus of *T. b. rhodesiense* in eastern Uganda. Patients from the Democratic Republic of the Congo were from Mbuji-Mayi and surrounding villages. Purified nucleic acids from other pathogens, i.e., *Plasmodium falciparum*, *Leishmania donovani*, *Brucella melitensis*, *Mycobacterium tuberculosis*, and *Salmonella enterica* serovar Typhi, were obtained from other research groups at KIT (Royal Tropical Institute) Biomedical Research (Amsterdam, The Netherlands).

Negative controls. Venous blood (200 μl) on EDTA was obtained from 20 healthy human volunteers in Uganda (an area of endemicity) as well as from 24 healthy individuals in Amsterdam, The Netherlands, who had never visited a country where HAT is endemic.

In vitro-cultured parasites. *T. b. gambiense* (LiTat 1.3) parasites were cultured in vitro in GLSH (glucose-lactalbumin-serum-hemoglobin) medium at 28°C. The parasites were subcultured weekly and harvested, and the parasite load was established using a Bürker counting chamber. The culture was centrifuged, and the parasite pellet was resuspended in phosphate-buffered saline to achieve a concentration of 10^5 parasites per μl of phosphate-buffered saline. Nucleic acid extracted from the in vitro-cultured parasites was used for making serial dilutions as well as for spiking blood.

Spiked blood. Blood on EDTA spiked with *T. b. gambiense* (LiTat 1.3) parasites was used throughout the development of the assay and for the estimation of its lower detection limit. A 10-fold dilution series of parasites ranging from 10,000 to 10 parasites per ml of blood was made in freshly collected naïve human blood. Nonspiked blood was used as a negative control.

Nucleic acid extraction. Nucleic acid was extracted as described by Boom et al. in 1990 (5), with some modifications. To each sample, 1.2 ml of guanidinium isothiocyanate cell lysis buffer (L6) was added, followed by 40 μl of a silica suspension, and the solution was mixed at room temperature for 5 min. After centrifugation, the supernatant was discarded; the pellet was washed twice with 1 ml of L2 wash buffer, twice with 1 ml of 70% ethanol, and once with 1 ml of acetone; and the pellet was air dried at 56°C for 5 min. Nucleic acids were eluted in 50 μl nuclease-free water during a 5-min incubation at 56°C and were stored at −20°C.

Production of in vitro RNA. In vitro internal-control RNA was made by site-specific mutagenesis as described previously (23). The internal-control RNA sequence (GGATTCCTTGCTTTTCGCGCTTAGGTCCACTAAGGTACCCAGCAGGTCTGTGATGCTCCTCAATGTTCTGGGCGACACGCGCACTACAATGTCAGTGAGAACAAGAGTCCGAGCGGCACT) is amplified by the same primers that target *T. brucei* 18S rRNA but contains a short internal modified sequence (underlined). The internal-control RNA was produced using an SP6 transcription kit (Ambion, Austin, TX). The internal-control RNA was included in the NASBA assay to check for amplification inhibition.

Primers and probes. (i) NASBA primers. The primer sequences used in this study were based on the target sequence of the 18S rRNA gene identified by Mugasa et al. (17), who designed forward primer TrypnasF7 (5′-GGATTCCTTGCTTTTCGC-3′) and reverse primer Trypnas6T7rev (5′-AATTCTAATACGACTCACTATAGGGAGAAGGCTCGGACTCTTGTTCTC-3′), containing the T7 polymerase binding site (underlined). However, in the current study, the forward primer was modified by the addition of a generic tail (GATGCAAGGTCGCATATGAG) at the 5′ end.

(ii) OC probes. Two probes biotinylated at the 5′ end were designed for the specific capture of the *T. brucei* amplicon (5′-GCAAGGTGAGATTTTGGGCA-3′) and the internal-control RNA amplicon (5′-CGCTTAGGTCCACTAAGGTACCC-3′), respectively. Amplification was detected with an internal probe (5′-CAGGTCTGTGATGCTCCTCAATG-3′), which is complementary to a sequence common to both amplicons. The detection probe was labeled with gold colloid particles by the procedure described in European patent WO 2004/099438A1 (20). A probe complementary to the detection probe (5′-CATTGAGGAGCATCACAGACCTG-3′) was used as a migration control on the dipstick.

NASBA. The NASBA reaction (also known as "self-sustained sequence replication") uses RNA targets to exponentially produce a large amount of reverse cRNA (9). The entire process needs two primers and three enzymes, allowing first the production of double-stranded DNA (dsDNA) from the RNA target by avian myeloblastosis virus reverse transcriptase (RNA⇒cDNA and single-stranded DNA⇒dsDNA) and RNase H (cDNA⇒single-stranded DNA) and then a continuous polymerization of target cRNA by the T7 DNA-dependent RNA polymerase (dsDNA⇒RNA) using newly formed dsDNA as the template. The amplification reaction is performed at 41°C and can de done without the interference of DNA (9).

Prior to NASBA, nucleic acid extracted from each blood and CSF sample was diluted in ultrapure water 1:5 and 1:10, respectively, to reduce the amount of amplification inhibitors that may be present in the samples. The NASBA reaction was performed using a NucliSens basic kit for amplification (comprising the three enzymes needed for replication: avian myeloblastosis virus reverse transcriptase, RNase H, and T7 RNA polymerase) according to the manufacturer's instructions (Biomérieux) in a 10-μl total reaction volume with KCl at a final concentration of 80 mM and containing 20 pmol/μl of the primers and 10^6 molecules of in vitro control RNA. The reaction mixture was incubated in a 0.5-ml tube with 2.5 μl RNA extract at 65°C for 2 min and subsequently at 41°C for 2 min. The isothermal amplification took place for 90 min at 41°C in a heat block. All samples were tested three times for reproducibility.

Design of the oligochromatographic stick. The OC dipstick (Fig. 1) is double-sided, with a polymer (plastic) support backing (*a*). On either side of the support, several membranes and absorbents regulate the flow and allow sequential hybridization. (i) The lower absorbent pad (*c*) is impregnated with the detection probe coupled to gold particles (probe conjugate). The probe conjugate is dried in the lower absorbent; it will be solubilized when the OC dipstick is placed in the NASBA product mixed with running buffer. The control side contains the probe conjugate specific for the internal-control RNA, while the test side contains the probe conjugate specific for the target (*T. brucei*). (ii) The intermediate nitrocellulose membrane (*b*) contains two capture zones on each side. The lower capture zones (*d*) allow the concentration of the nucleic acid detected. They are coated with the two specific capture probes binding the *T. brucei* amplicon on the test side (*d1*) and the internal-control RNA amplicon on the control side (*d2*). The upper capture zones, or migration control lines (*e*), allow the validation of the migration by capturing the excess probe conjugates on both sides. The capture elements on these zones are oligonucleotides complementary to the probe conjugates. (iii) The upper absorbents (*f*) allow the migration of liquid on the nitrocellulose membrane to continue by absorbing the excess.

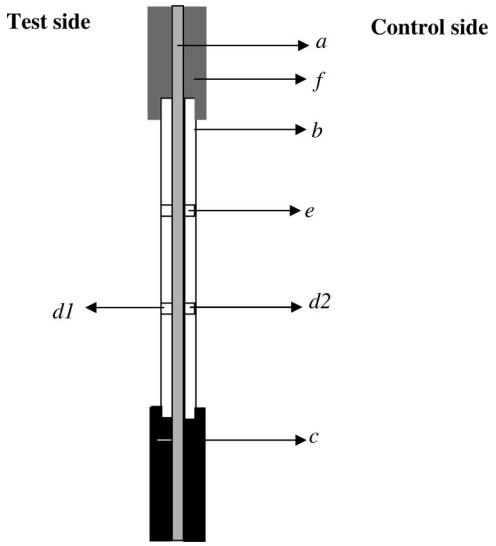

FIG. 1. Design of oligochromatographic dipstick (side view). *a*, polymer backing; *b*, intermediate nitrocellulose membrane; *c*, lower absorbent pad impregnated with the probe conjugate; *d*, lower capture zones; *e*, migration control lines; *f*, upper absorbents. (Test side) During migration, if the sample is positive, the *T. brucei* gold probes hybridize with the *T. brucei* amplicons that will accumulate on the lower capture zone (*d1*), resulting in a visible colored line on the test side of the stick. (Control side) During migration, the gold-labeled control probes hybridize with the internal-control RNA amplicons that will accumulate on the lower capture zone (*d2*), thereby forming a visible colored line on the control side of the stick. The unbound gold detection probes hybridize with the complementary oligonucleotides at the upper capture zone (migration control lines). The OC test is considered invalid when one of the migration control lines is absent. The NASBA test is considered invalid when both the test line (*d1*) and the internal control line (*d2*) are absent.

Detection of amplification by OC. NASBA products were detected by OC in an assay tube preheated at 55°C. Four microliters of NASBA product was mixed with 76 μl of migration buffer preheated at 55°C, and the OC dipstick was then dipped into the solution. The assay tube was sealed with a cap, and after 5 to 10 min, the OC dipstick was read on both faces (sides).

RESULTS

Analytical sensitivity and specificity. The lower detection limit of the *T. brucei* NASBA-OC assay was 10 parasites per ml of spiked blood, and occasionally the dipstick was able to detect 1 parasite per ml of in vitro-cultured parasites (serial dilution of RNA extracted from parasite culture). The NASBA-OC test developed has a specificity of 100%. All nontarget pathogens included in the study tested negative by the *T. brucei* NASBA-OC assay. Control blood samples from healthy individuals in areas where HAT is endemic ($n = 20$) and in areas where HAT is not endemic ($n = 24$) also tested negative by the NASBA-OC dipstick test.

***T. brucei* NASBA-OC assay on samples from HAT patients.** A total of 122 clinical samples (65 blood and 57 CSF samples) collected from HAT patients were available for analysis. Two blood samples and six CSF samples (all from Uganda) could not be analyzed by the NASBA-OC assay, because the test result was invalid due to problems with RNA extraction. The remaining 114 samples were analyzed by the NASBA-OC assay, and test results were compared to the results of initial microscopy as the reference test. All NASBA-OC tests were repeated three times, and the outcomes of testing were consistent on all occasions.

TABLE 1. Results of *T. brucei* NASBA-OC and microscopy performed on 63 blood samples collected from confirmed HAT patients

Result by NASBA-OC	No. of specimens with the following result by microscopy:		Total
	Positive	Negative	
Positive	35	11	46
Negative	1	16	17
Total	36	27	63

Both microscopy and the *T. brucei* NASBA-OC assay identified 35 blood samples (19 from Uganda and 16 from the Democratic Republic of the Congo) as positive, but both failed to detect parasites in 16 samples (1 from Uganda and 15 from the Democratic Republic of the Congo). In addition, the *T. brucei* NASBA-OC assay on blood found 11 samples positive (8 from the Democratic Republic of the Congo and 3 from Eastern Uganda) that were not detected by microscopy but failed to detect 1 sample (from the Democratic Republic of the Congo) that was positive by microscopy (Table 1). Compared to the composite standard for a confirmed HAT case (i.e., clinical suspicion plus a sample positive either by direct microscopy or by microscopy after concentration with a microhematocrit centrifugation or a mini-anion-exchange centrifugation technique), the NASBA-OC assay on blood samples had a sensitivity of 73.0% (95% confidence interval, 60 to 83%) and standard microscopy had a sensitivity of 57.1% (95% confidence interval, 44 to 69%).

Thirty-nine CSF samples were found parasite positive by both methods, but both failed to detect the presence of *T. brucei* in one sample (from a patient from the Democratic Republic of the Congo). In addition, microscopy detected parasites in five CSF samples (all from the Democratic Republic of the Congo) that the *T. brucei* NASBA-OC assay failed to detect. On the other hand, six microscopically negative CSF samples (one from a patient from the Democratic Republic of the Congo and five from Eastern Uganda) were positive by the *T. brucei* NASBA-OC assay (Table 2). Compared to the composite standard, the NASBA-OC assay on CSF samples had a sensitivity of 88.2% (95% confidence interval, 75 to 95%) and

TABLE 2. Results of *T. brucei* NASBA-OC and microscopy performed on 51 CSF samples collected from confirmed HAT patients

Result by NASBA-OC	No. of specimens with the following result by microscopy:		Total
	Positive	Negative	
Positive	39	6	45
Negative	5	1	6
Total	44	7	51

TABLE 3. Comparison of some test characteristics of methods generally employed for the diagnosis of HAT

Technology	Source or reference used to estimate parameters	Detection limit (parasites per ml of sample processed)	Sensitivity (%) (reported range)	Specificity (%) (reported range)	Avg processing time (excluding nucleic acid extraction)	Equipment needed	Estimated costs (€) (excluding labor costs)	Remarks
Microscopic examination of blood	6, 17; this study	1,000–10,000	57–100		20–30 min	Microscope and possibly concn equipment	1.00	Detection limit and test sensitivity and specificity can be enhanced by concn of parasites, but this will increase processing time and costs
Microscopic examination of CSF	6	Difficult to determine, because parasites tend to disintegrate after sample collection	86–100		30–60 min	Microscope and possibly concn equipment	1.00–3.00	Detection limit and test sensitivity and specificity can be enhanced by concn of parasites, but this will increase processing time and costs
Serology (card agglutination test for trypanosomiasis)	6, 11	Antibody test; not quantifiable	87–98	95	10 min	Agglutination card and materials for blood collection	1.00	Intended as a screening test for *T. brucei gambiense* only
PCR systems	4, 7, 13, 19	6–100	87–100	97–100	2–4 h	Real-time PCR machine or thermocycler, combined with ethidium bromide gel system or OC sticks	2.60–4.75	Costs range from those of standard PCR to those of quantitative real-time PCR; processing time depends on the readout system (gel electrophoresis vs real-time system)
NASBA systems	17, 23, 25	10–100	73–100	97–100	2 h	Water bath or heat block; real-time reader or ECL detection system	5.20	Costs of NASBA are higher due to costs of amplification reagents; cost estimate is based on use of a real-time NASBA assay
NASBA-OC	This study	10	73–89	100	90 min	Water bath or heat block; OC sticks	4.00–4.50	Costs are reduced because of the simplified readout system

standard microscopy had a sensitivity of 86.2% (95% confidence interval, 64 to 88%).

DISCUSSION

In developing countries, particularly in situations where diseases are diagnosed under harsh field conditions, simple and fast diagnostic tests are much needed (22). The aim of the present study was to develop a fast and easy-to-use molecular diagnostic tool, simplified at the readout level of the test, for the detection of the NASBA product of *T. brucei* 18S rRNA. Oligochromatographic detection of the NASBA product, which is described in this study, shows great potential for use in the poorly equipped laboratories characteristic of regions of HAT endemicity. The *T. brucei* NASBA-OC test has a lower detection limit of 10 parasites/ml of blood or 2 parasites per 200-μl sample of blood collected from patients for HAT diagnosis. This detection limit is similar to that achieved by Mugasa et al. (17), who used a sophisticated IQ5 real-time analyzer for the detection of *T. brucei* after NASBA. This means that the simplification of the detection system does not undermine the sensitivity of the test. The detection tool in this study was superior to blood microscopy in that it detected parasites in nine samples that were regarded negative by microscopy. However, the microscopically positive blood sample that was not detected by the OC test cannot be explained. Both methods failed to detect trypanosomes in 16 blood samples, of which 15 were from the Democratic Republic of the Congo (*T. b. gambiense* HAT) and only 1 sample was from a *T. b. rhodesiense* patient in eastern Uganda. Parasite detection in blood is frequently successful in cases of *T. b. rhodesiense* infection because of the permanent parasitemia. However, it is very difficult in *T. b. gambiense* infection, in which few parasites are present in the peripheral circulation at times other than the periods of cyclic parasitemia (6, 21). Thus, if the blood of *T. b. gambiense* HAT patients is sampled outside of the period of cyclic parasitemia, even a sensitive detection tool such as the *T. brucei* NASBA-OC test may not detect the trypanosomes. In such instances, periodic blood sampling may be advised in order to increase the rate of detection of cases (6). In some cases, individuals have been reported to remain apparently without parasites for several months, but repeated searches for parasites yielded positive results (11).

In the management of HAT, one of the most important tasks is to accurately distinguish the late, encephalitic stage of HAT from the early, hemolymphatic stage. Accurate staging of HAT is critical because failure to treat a patient with central nervous system involvement will lead inevitably to death from the disease, yet inappropriate treatment of an early-stage patient carries a high risk of unnecessary drug toxicity (16). Various molecular techniques have been developed to detect trypanosomes in blood for HAT diagnosis (4, 7, 14, 17, 19), but a handful have been developed for the detection of parasites in CSF from HAT patients. A CSF PCR was developed to detect trypanosome DNA for the diagnosis of HAT, but despite its high sensitivity (96%), the assay has been documented to have reproducibility problems (24). This casts doubt on its value for therapeutic decision making (12); moreover, PCR is not readily available under field conditions. With the shortfalls of the CSF PCR, there is still an urgent need for a test that can be reliably used in staging and treating HAT.

In this study, the *T. brucei* NASBA-OC test that was developed was used to detect parasite RNA in CSF from HAT patients and had a reproducibility of 100%, making this assay reliable and valuable for deciding the course of treatment. The *T. brucei* NASBA-OC assay had a sensitivity of 88.6% and detected parasites in six microscopically negative samples but failed to detect them in five samples that were positive by microscopy. However, the results of the *T. brucei* NASBA-OC assay are promising given the difference in the volumes of CSF samples processed by the two methods under comparison. For microscopic detection, 4 ml of CSF was centrifuged and the resulting sediment examined under the microscope. On the other hand, only 200 μl of CSF was used to extract nucleic acid for NASBA.

During this study, two blood samples and six CSF samples that were microscopically positive gave invalid results in the NASBA-OC assay. The reason for the invalidity remains to be understood, but in general, invalid test samples are caused by inhibitory substances in the sample due to improper RNA isolation rather than by the NASBA itself. Therefore, a validation study should be carried out on larger samples of both blood and CSF to elucidate this observation.

A comparison between different direct parasite detection methods for the diagnosis of HAT is presented in Table 3. Although microscopic examination of body fluids is cheap and rather quick, these methods have limited sensitivity/specificity, and misdiagnosis is a considerable risk. Molecular methods are superior in terms of sensitivity and specificity. PCR-based methods are in general cheaper than NASBA-based diagnostics but may require more time and equipment. Notwithstanding, based on the results obtained in this study coupled with the ease and speed of the NASBA-OC assay, we believe that this assay offers an alternative tool for diagnosing and staging HAT that can be employed in mid-level laboratories characteristic of developing countries that are ravaged by HAT.

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